REPORT
25th SOCIAL RETURN OF THE RESEARCH
CANCER

EPIGENETIC CHARACTERISATION OF CHOLANGIOCARCINOMAS

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1. Summary of the project

GOALS

In this project, we proposed using our unique collection of cholangiocarcinoma (CCA) patient-derived models to characterize them molecularly and epigenetically. We planned to perform an integrative analysis to characterize CCA molecularly and epigenetically and understand the relation between the genetic and epigenetic architecture of CCAs. Moreover, we planned to test different inhibitors in IDH1/2 CCA models and study the biology behind this activity. This may lead to identifying potential therapeutic biomarkers and/or novel therapeutic strategies.

SPECIFIC AIMS

Establishment and characterization of patient-derived xenografts (PDX) from CCAs IDH1 wild-type and mutant.

1. Creation of a reference epigenome of CCA_PDXs IDH1 wild-type and mutant models and transcriptome profiling.
2. Evaluate the efficacy of different treatments in IDH1 wild-type and mutant models of CCA_PDXs.
3. Identify potential biomarkers to predict the response to this treatment and improve patient stratification.

METHODS

AIM 1: Establishment and characterization of patient-derived xenographs (PDX) from CCAs IDH1 wild-type and mutant.

AIM 2: Reference epigenome generation from CCA_PDXs and transcriptome profiling

- Samples from 6 CCA_PDX IDH1^{wt} 6 CCA_PDX IDH1^{mut} will be used to perform ChIP-seq.
- To characterize the mutational landscape of CCA PDXs and compare them with tumor biopsies.
- To generate reference epigenomes from CCA PDXs consisting of DNA accessibility by ATAC-seq (assay for transposase-accessible chromatin).
- Integrative analysis to link changes in gene expression to epigenetic changes in the nearby chromatin regions.

AIM 3: Evaluate the efficacy of different treatments in IDH1 wild-type and mutant models of CCA_PDXs

- IDH1 wild-type and mutant CCA_PDXs models will be treated ex-vivo with platinum-based chemotherapy.
- To obtain gene expression profiles by RNA-seq in all generated models, with specific attention to platinum-sensitive (PtS) in combination with PARP-inhibitors(PtS_PARPiR (resistant) and PtS_PARPiS (sensitive)).

AIM 4: Identify potential biomarkers to predict the response to this treatment and improve patient stratification

- RNA-seq and DNA methylation in the PDX_ $IDH1^{mut}$ setting compared with the PDX_ $IDH1^{wt}$ setting will give us biomarkers candidates which qRT-PCR and IHQ will validate in tumor samples.
- We will identify biomarkers that allow us to stratify patients based on their differential expression, methylation, and/or accessibility. We will perform several validations, such as qRT-PCR and immunohistochemistry, on tumor samples from patients.

2. Results

Part of the results are still being published (CONFIDENTIAL).

Transcriptomic profiling of CCA_PDXs

RNA-seq data analysis of CCA_PDX samples using principal component analysis (PCA) and unsupervised hierarchical clustering revealed two distinct clusters based on IDH1 mutation status. Cluster 1 (*IDH1*^{mut} and *IDH1*^{wt} PDX133) showed enrichment in metabolic processes, such as fatty acid metabolism or the biosynthesis of bile acids. In contrast, Cluster 2 (*IDH1*^{wt}) is enriched in developmental pathways, such as WNT-β-catenin and notch, immune-related pathways, and epithelial-to-mesenchymal transition (EMT) factors.

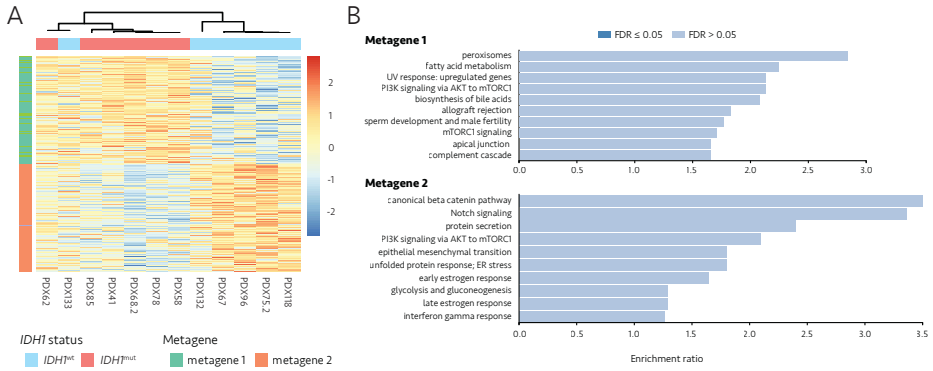

Figure 1. Unsupervised clustering of RNA-seq data separated CCA_PDX samples according to *IDH1* mutation status.(A) Unsupervised clustering of CCA_PDXs based on RNA-seq data.**(B)** Gene set enrichment of the two metagenes. FDR, false discovery rate.

<u>Differential expression (DE) analysis revealed a significant downregulation of gene expression and overlapping with metagenes.</u>

DE analysis comparing *IDH1*^{mut} and *IDH1*^{wt} CCA_PDXs revealed 575 dysregulated genes in *IDH1*^{mut} tumors, from which 447 were downregulated. Gene set enrichment analysis (GSEA) using the Hallmark database showed downregulation of pathways related to hypoxia, estrogen response, EMT, and the immune system in *IDH1*^{mut} CCA_PDXs. The comparison between the genes identified in the DE analysis and the metagene sets from the unsupervised clustering analysis revealed a significant overlap ($p < 2.2 e-16$), suggesting an essential contribution of differentially expressed genes to the sample clustering.

<u>The expression of the immune-related genes is downregulated in *IDH1*^{mut} CCA_PDXs.</u>

We focused our analysis on comparing the expression of immune-related genes and pathways in our *IDH1*^{mut} and *IDH1*^{wt} CCA_PDXs."Inflammatory response," interferon-alpha (IFNα), and interferon-gamma (IFNγ) signatures showed trends to be negatively enriched in our *IDH1*^{mut} CCA_PDXs. The comparison between *IDH1*^{mut} and *IDH1*^{wt} CCA_PDXs revealed a notable decrease in the expression of genes related to immune pathways, including chemokines, cytokines, and interleukins. Among the downregulated genes in *IDH1*^{mut} CCA_PDXs, stimulator of interferon genes (*STING1)* stood out, known for regulating the transcription of host defense genes like type I interferons (IFNs) and pro-inflammatory cytokines. STING1 plays a crucial role in recruiting immune cells to tumor sites and is increasingly recognized for its importance in enhancing immune responses against tumors. We used a 47-gene Interferon

signature (IFNsign) to assess our samples, which combines genes from IFN-alpha and IFN-gamma response sets. High IFNsign expression (IFNsign^{high}) is linked to increased immune infiltration and a proinflammatory microenvironment in PDAC. Clustering based on IFNsign genes separated samples into *IDH1*^{wt} and *IDH1*^{mut} enriched groups. IDH1wt*IDH1*^{wt} CCA_PDXs tended to have higher IFNsign scores than *IDH1*^{mut} ones. To confirm this, we selected eight interferon pathway-related genes previously identified as differentially expressed between groups and used RT-qPCR to validate their expression in all the PDXs in our bank. Our findings were consistently maintained in the expanded sample cohort, and our data demonstrated a decreased immune gene expression in *IDH1*^{mut} CCA_PDXs.

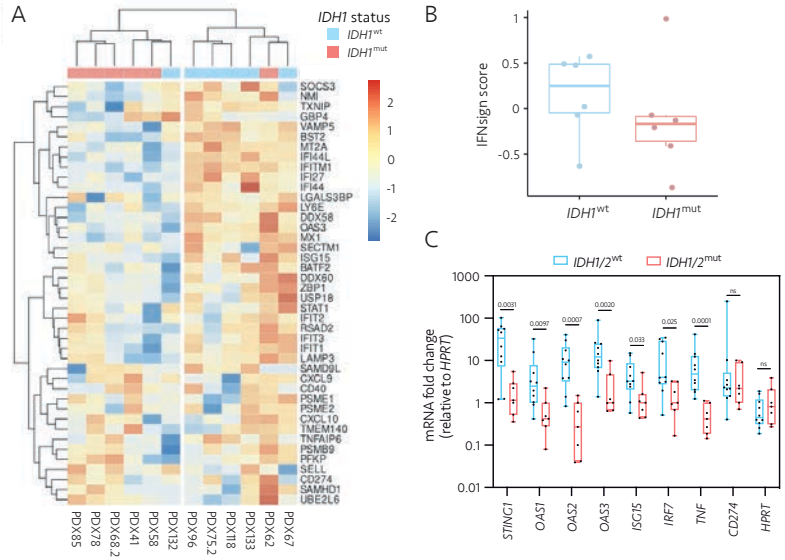

Figure 2. Genes involved in the interferon signaling are downregulated in *IDH1*^{mut} CCA_PDXs. **(A)** Heatmap showing the unsupervised clustering of CCA_PDXs based on the differential expression of IFN sign genes. **(B)** IFNsign score in *IDH1*^{wt} and *IDH1*^{mut} CCA_PDXs. **(C)** RT-qPCR analysis of interferon-related gene expression in our CCA_PDXs collection.

DNA methylation profiling of CCA_PDXs

We analyzed DNA methylation in our CCA_PDX collection using the Illumina Human Methylation EPIC BeadChip. The differences in DNA methylation of individual CpG sites revealed that most changes were methylation gains in *IDH1*^{mut} CCA_PDXs. Through GSEA analysis, we found that CpG associated with genes that are involved in pathways such as EMT, hypoxia, and estrogen response appeared differentially methylated between *IDH1*^{wt} and *IDH1*^{mut} CCA_PDXs. Importantly, when we focused on promoter methylation, we found that CpGs involved in IFNγ response appeared to be

differentially methylated between the two groups. We demonstrated that CpGs are significantly more methylated in *IDH1*^{mut} CCA_PDXs, suggesting that hypermethylation of immune-related gene promoters is linked to the downregulation of these genes in these PDXs.

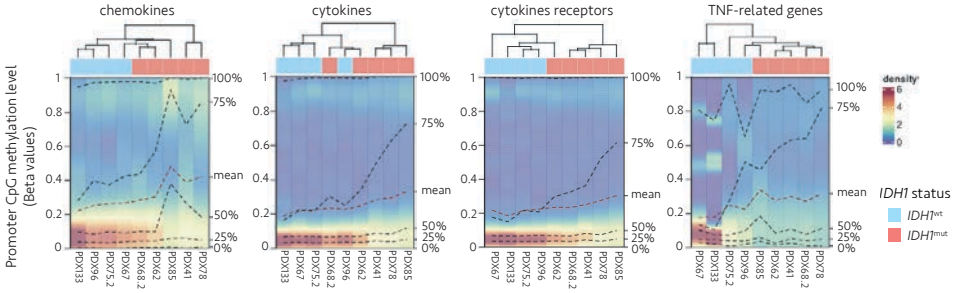

Figure 3. Promoter CpGs of immune-related genes are hypermethylated in *IDH1*^{mut} CCA_PDXs, compared to *IDH1*^{wt}. Density heatmaps showing the promoter CpG methylation levels found in CCA_PDXs.

<u>Mapping of the tumor immune microenvironment (TIME) of CCA_PDXs by multiplexing immunohistochemistry</u>

Based on our transcriptomic and DNA methylation studies on CCA_PDXs, we hypothesized that in *IDH1*^{mut} CCA_PDXs, 2-HG produced by the mutant IDH1 enzyme could be leading to a DNA hypermethylation of specific immune-related gene promoters. We conducted multiplexing immunohistochemistry (mIHC) on biopsy samples from 8 patients with advanced CCA; four of these samples were *IDH1*^{mut,} and others were *IDH1*^{wt}. The results showed that *IDH1*^{mut} samples exhibit a slightly elevated immune cell infiltration. In the area within the tumor, samples show a higher concentration of CD163^{+} cells than CD3^{+} cells, irrespective of *IDH1* mutation status. Results indicate that patients with *IDH1*^{mut} CCA might experience problems with T cell activation rather than T cell infiltration.

<u>Therapeutical strategies to boost the immunogenicity of *IDH1*^{mut} CCAs</u>

Our findings indicate that reduced expression of immune-related genes, possibly due to excessive DNA methylation of their promoter regions, could be associated with an altered tumor immune microenvironment of *IDH1*^{mut} CCAs that may negatively affect the effectiveness of T cell tumor infiltrates. We propose that inhibiting either the mutant IDH1 enzyme or DNA methyltransferases (DNMT), which are responsible for

DNA methylation, could reverse this immunosuppressed phenotype and make *IDH1*^{mut} CCAs more responsive to immuno-oncology.

CCA cell lines recapitulate the phenotype observed in CCA_PDXs

We verified whether these cell models recapitulate the phenotype previously observed in our CCA_PDX models. As expected, both *IDH1*^{mut} cell lines showed higher 2-HG levels than the *IDH1*^{wt} cells. Accordingly, higher levels of the DNA methylation mark 5-methylcytosine (5mC) were found in *IDH1*^{mut} Snu1079 and RBE cells. Importantly, we also found that one of the top downregulated genes identified in the *IDH1*^{mut} CCA_PDXs, *STING1*, was downregulated in *IDH1*^{mut} Snu1079 and RBE cell lines, both at the mRNA and the protein level.

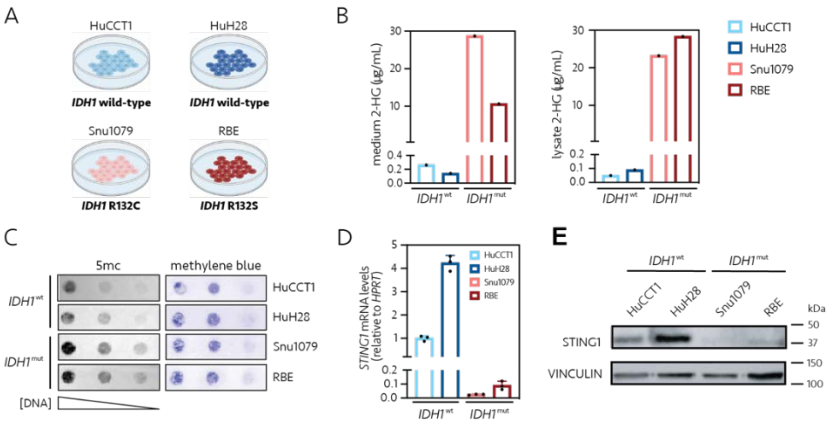

Figure 4. Characterization of the four CCA cell line models. **(A)** *IDH1* mutation status of four CCA cell line models. **(B)** Quantification of 2-HG by LS-MS. **(C)** 5mC levels evaluation by Dot Blot. **(D)** RT-qPCR analysis of *STING1*. *HPRT* expression was used as a housekeeping gene for normalization. mRNA data are presented as mean ± s.d. **(E)** Determination of STING1 protein levels by Western Blot. VINCULIN was used as the loading control.

Treatments increasing STING expression in *IDH1*^{mut} CCA cell lines

We treated CCA cells with a clinically tested DNA demethylating agent, decitabine. Its application decreased 5mC in both *IDH1*^{wt} and *IDH1*^{mut} cell lines. Nevertheless, the upregulation of *STING1* was observed solely in *IDH1*^{mut} CCA cell lines and not in *IDH1*^{wt} cells. Treating DNA demethylating agents can increase *STING* expression in *IDH1*^{mut} CCA cell lines. To verify whether the inhibition of mutant IDH1 could lead to an increase of immune-related genes, we treated *IDH1*^{mut} cell lines Snu10790 and RBE with the mutant IDH1 inhibitor, ivosidenib. Accordingly, reduced 5mC levels were found in *IDH1*^{mut} cell lines treated with ivosidenib for 120 hours. We treated Snu1079

and RBE constantly with ivosidenib (5 μM), and these new lines were used for further functional studies.

Both DNA demethylating agent and mutant IDH1 inhibitor treatments have been shown to decrease 5mC levels and increase *STING* expression. We performed genome-wide transcriptomics and DNA methylation studies to verify whether the two potential strategies can enhance immune gene expression.

HER2-T cell bispecific antibody can be used to study T cell activation

We tested if giving decitabine and ivosidenib to *IDH1*^{mut} CCA cells would affect the activation of T cells. We conducted a flow cytometry test to confirm the expression of HER2 in our CCA cell lines under basal conditions and upon indicated treatments. The results showed that all four CCA cell lines expressed HER2, and the variation in its expression upon decitabine or ivosidenib treatments does not play a significant role in T cell activation. We exposed CCA cell lines to 5 μM decitabine for 24 or 48 hours. Then we refreshed the culture medium to remove any remaining decitabine and co-cultured them with PBMCs from healthy donors for an additional 72 hours with the presence of 100 pM HER2-TCB. We measured T cell activation and cytotoxicity using the late activation marker CD25 and the cytotoxic marker Granzyme B (GZMB). The results of the study showed that when HER2-TCB treatment was not administered, there was little activation of T cells (%CD25$^+$ (of CD3$^+$ cells)) and cytotoxicity (%GZMB$^+$ (of CD3$^+$ cells)). However, when HER-TCB was present (at a concentration of 100 pM), co-culturing decitabine-pretreated *IDH1*^{mut} Snu1079 and RBE cells with PBMCs led to a significant increase in the activation of T cells and cytotoxic GZMB expression. In contrast, when decitabine-pretreated *IDH1*^{wt} HuCCT1 and HuH28 cells were co-cultured with PBMCs, the activation of T cells and cytotoxicity were minimal.

We examined the T cell activation and cytotoxicity markers in the CD8$^+$ population and the T cell activation in the CD4$^+$ population. We found that, as compared to decitabine pretreated *IDH1*^{wt} cells (HuCCT1 and HuH28), *IDH1*^{mut} cells (Snu1079 and RBE) treated with decitabine increased the activation and the cytotoxicity of CD8$^+$ T cells under the presence of HER2-TCB more significantly. In addition, *IDH1*^{mut} cells pre-treated with decitabine also increased the activation of CD4$^+$ T cells under the presence of HER2-TCB. Notably, CD4$^+$ helper T cells play a critical role in supporting effector CD8$^+$ functions and directing a sustained immune response against tumors.

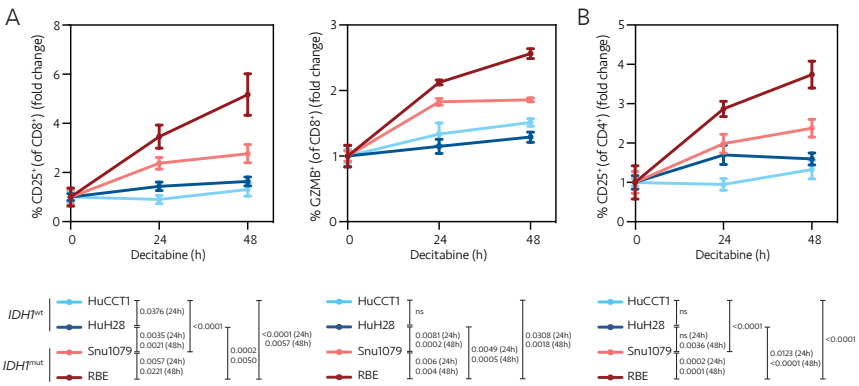

Figure 5. Treatment with decitabine increases cytotoxic CD8⁺ and helper CD4⁺ T cell activation only in co-cultures with *IDH1*^{mut} CCA cells. CCA cells previously treated with 5μM decitabine were co-cultured with PBMCs at a 1:1 ratio and 100pM of HER2-TCB for 72h prior to marker evaluation. **(A)** Flow cytometry analysis of CD25 late activation marker (left) and Granzyme B (GZMB) cytotoxicity marker (right) in CD8⁺ T cells. **(B)** Flow cytometry analysis of CD25 late activation marker in CD4⁺ T cells. The percentage of positivity is represented as the fold change compared to the untreated control for each cell line. Data are presented as mean ± s.d., n=3 independent replicates, and were analyzed by two-way ANOVA multiple comparisons with Tukey correction.

Next, we evaluated whether mutant IDH1 inhibitor ivosidenib treatment of *IDH1*^{mut} cells could increase T cell activation and cytotoxicity with the presence of HER2-TCB and found no significant changes in T cell activation and toxicity.

3. Relevance with possible future implications

Our proposal will systematically address the specific effects of *IDH1* mutations on the immune cell contents of CCA and its dynamic changes during treatment. These data will provide a wealth of opportunities for clinical solutions for patients with CCAs: 1) Future drug development: elucidating the molecular details of the mechanism will provide a pharmacological solution to treat incurable CCA; 2). Biomarkers and immunotherapy: having a detailed map of gene expression in tumor samples from our results will support discovering new biomarkers and developing immunotherapy strategies with tremendous clinical potential; and 3) Improved decision-making based on predictive biomarkers that will offer a novel opportunity to enhance therapeutic decision-making and thus, improve the efficacy of immunotherapies.

4. Scientific bibliography generated

Publications

1. **Yanez-Bartolome M, Macarulla T, Tian TV**. The potential of patient-derived organoids in precision medicine of biliary tract cancer. Cell Rep Med. 2023;4(11):101294. Epub 2023/11/23. doi: 10.1016/j.xcrm.2023.101294. PubMed PMID: 37992681.

2. **Serra-Camprubi Q, Verdaguer H,** Oliveros W, **Lupion-Garcia N,** Llop-Guevara A, Molina C, Vila-Casadesus M, Turpin A, Neuzillet C, Frigola J, **Querol J, Yanez-Bartolome M**, **Castet F, Fabregat-Franco C, Escudero-Iriarte C**, Escorihuela M, Arenas EJ, Bernado-Morales C, Haro N, Giles FJ, Pozo OJ, Miquel JM, Nuciforo PG, Vivancos A, Mele M, Serra V, Arribas J, Tabernero J, Peiro S, **Macarulla T, Tian TV**. Human Metastatic Cholangiocarcinoma Patient-Derived Xenografts and Tumoroids for Preclinical Drug Evaluation. Clin Cancer Res. 2023;29(2):432-45. Epub 2022/11/15. doi: 10.1158/1078-0432.CCR-22-2551. PubMed PMID: 36374558; PMCID: PMC9873249.

PhD thesis

Queralt Serra, Ä journey towards the identification of novel therapeutical strategies for advanced cholangiocarcinoma with IDH1 mutations, 27 July 2023. Cum Laude.